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Phenotypic Characterization of Lettuce Dwarf Mutants and Their Response to Applied Gibberellins[1]

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ABSTRACT

Four monogenic, recessive dwarf mutants of lettuce (*Lactuca sativa* L.), previously isolated from a population induced by ethyl methanesulfonate, were compared with the normal genotype (E-1) for plant height, weight, leaf area, as well as hypocotyl length and root length. These nonallelic dwarfs (*dwf1*, *dwf2*, and *dwf3*) exhibited reduced hypocotyl length, smaller, dark green leaves, and reduced stem length. Another mutant, *dwf2*^{l}, allelic with *dwf2*, exhibited an intermediate phenotype. Epidermal cells on hypocotyls and mature leaves were counted for both normal E-1 and *dwf2* plants. The total number of epidermal cells per unit area for hypocotyls and for leaves from these plants was very similar, implying the dwarf's smaller size was due to an inhibition of cell expansion and not due to decreased cell divisions. Both dwarf and normal hypocotyls elongated normally in response to exogenous gibberellin A_3 (GA_3). In the rosette stage, only E-1 and *dwf2*^{l} responded similarly to lower concentrations of GA_3, while the other dwarfs required higher concentrations to respond. Hypocotyls of *dwf2* and E-1 elongated equally with applied *ent*-kaurenol, *ent*-kaurenoic acid, GA_{53}-aldehyde, GA_{53}, GA_{19}, GA_{20}, and GA_1 indicating that the biochemical block in *dwf2* occurs at a very early step in the GA-biosynthetic pathway.

GAs[3] have been shown to regulate stem elongation in dicotyledonous rosette plants (3, 7, 10, 25, 26). This is evident from the increased stem growth in response to certain environmental stimuli (7) and to GA treatments (3) and from the response of dwarf mutants (18). Bukovac and Wittwer (2, 28) showed that stem elongation in the cumulative long day plant lettuce was stimulated by exogenous GAs. The highly responsive nature of normal lettuce to applied gibberellins has been studied and used in hypocotyl elongation bioassays for many years (1, 21–23). It has been inferred that the early-13-hydroxylation pathway of gibberellin biosynthesis operates in lettuce (14). Based on these studies, it seems likely that stem elongation in lettuce is regulated by increased GA biosynthesis via the early-13-hydroxylation pathway.

GA-responsive dwarf mutants have been reported in maize (15, 16), pea (5, 6, 19), *Arabidopsis thaliana* L. Heynh. (9, 10, 29), rice (11, 12), tomato (8), and others. Several of these dwarfs are deficient in endogenous GAs, while all of them respond to some degree to applied GA. This response by the dwarfs is often greater than the response of the corresponding normal genotype, though this is not so for pea (19) or tomato (8). Thus, dwarf mutants in several species appear to be more responsive to GA than their normal counterparts while a few others do not exhibit this trait.

In this paper we phenotypically characterize four single-gene, recessive dwarf mutants of lettuce and measure their response to exogenous GA_3. Hypocotyls of *dwf2* and the normal genotype were also measured in response to *ent*-kaurenol, *ent*-kaurenoic acid, GA_{53}-aldehyde, GA_{53}, GA_{19}, GA_{20}, and GA_1.

MATERIALS AND METHODS

Seedling Growth Conditions

The lettuce (*Lactuca sativa* L.) strains used in this study were normal E-1 (SC352), dwarf-1 (SC904), dwarf-2 (SC367), dwarf-4 (SC3515), and dwarf-8 (SC3670). All strains are single-gene mutants of E-1 and have a 60-d maturity, except for dwarf-1, which is a single gene mutant of E-3 and has an 80-d maturity (27). Seeds were surface-sterilized with 70% (v/v) ethanol, rinsed with sterile distilled water, followed by 0.5% (v/v) sodium hypochlorite, and rinsed again with sterile distilled water. They were then placed on two layers of Whatman No. 1 filter paper in 14 × 14 × 7 cm polypropylene containers, moistened with 10 mL of growth medium containing 5.0 mM KCl and 0.1 mM $CaCl_2$, covered with plastic wrap, and placed in an incubator at 23°C. Light was given as indicated.

Hypocotyl Elongation Response to GA_3

Seeds of each strain were surfaced sterilized and grown in polypropylene containers under the conditions described above with continuous light (30 μmol m^{-2} s^{-1}), for 36 h. Uniform seedlings were transferred to containers with 15 mL growth medium, plus different concentrations of GA_3 and returned to the incubator for 72 h.

Seedling Growth Bioassays

Twenty seeds of each strain were sown directly in Petri dishes onto moistened filter paper as described above, except that the germinating seedlings were placed in a 15°C chamber for 24 h and then transferred to 26°C for 5 d. Two different fluence rates of white light were used: half the seeds received

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[3] Abbreviations: GA, gibberellin; SC, accession number series used during dwarf isolation; M_2, second generation after mutation; EMS, ethyl methanesulfonate.

120 μmol m^{-2} s^{-1}, the other half received 45 μmol m^{-2} s^{-1}. At the end of the treatment, hypocotyl length, cotyledon length, and root length were measured.

Shoot Growth in Response to GA_3

Seeds of dwarfs and normal were sown directly in sterilized field soil, grown in a greenhouse. The plants were fertilized weekly with general purpose, water-soluble fertilizer (20N:8.7P:16.7K) (Peters Fertilizer Products, Fogelsville, PA) at half-strength. Three-week-old plants were treated with different concentrations of GA_3 and allowed to grow for an additional week. At 4 weeks, the stems and leaves of these plants were measured for response to GA_3.

Hypocotyl Elongation Response to 13-Hydroxy GAs

E-1 and dwarf-2 seedlings were germinated in droplets containing increasing concentrations of several 13-hydroxy gibberellins and their precursors. Individual dry seeds of each line were placed in 10 μL droplets containing growth medium plus one of the following: *ent*-kaurenol, *ent*-kaurenoic acid, GA_{53}-aldehyde, GA_{53}, GA_{19}, GA_{20}, and GA_1, at concentrations of 0.50 ng μL^{-1} to 50.0 ng μL^{-1} as indicated. Droplets (10 μL) were placed evenly spaced around the inner surface of a clean, dry, 150 mm diameter glass Petri dish. One dry seed was placed in each droplet, the dishes were then sealed with plastic film, and placed in a growth chamber at 23°C, with continuous light (30 μmol m^{-2} s^{-1}), for 48 h. At the end of this period, the entire solution had been imbibed by the germinating seedling. These seedlings were transferred to large clear plastic containers (35 cm × 25 cm × 10 cm) on two sheets of Whatman No. 3 filter paper moistened with growth medium. The boxes were sealed with plastic film and returned to the growth chamber for an additional 72 h. At the end of 5 d, seedling hypocotyl lengths were measured.

Leaf and Stem Measurements

Seeds of dwarfs and normal were sown directly into sterilized field soil in 10 cm × 10 cm × 10 cm plastic pots and thinned to five plants per pot after emergence. Plants were grown in a greenhouse and no fertilizer was supplied. All plants were measured for total stem height, total shoot height, area of the single largest leaf, average number of leaves per plant, and total leaf area at maturity, 63 d after sowing. Single largest leaves were measured at 49 d. Dwarf-1 (E-3 type) was measured 78 d after sowing. Leaf area was measured using a Delta-T Area Measuring System, type AMS with a CB Conveyor Unit (Decagon Devices, Inc., Pullman, WA).

Light Microscopy Studies

Fresh sections of dwarf-2 and normal E-1 hypocotyls and leaves were grown as above and prepared by first floating the sections on water, then placing them in an Azure A stain (0.1% Azure A mixed in Cellosolve, nine drops added to one drop 0.2 M $Na_2HPO_4 \cdot 7H_2O$ [pH 9.0]). The staining tissue was treated with microwave irradiation for 5 s, then rotated 90° and irradiated for another 5 s. Destaining was carried out with 100% ethanol, two or three times until excess stain was gone. Sections were mounted in Euparol.

RESULTS

Description of Dwarf Mutants

Four monogenic, recessive dwarf mutants were previously isolated among M_2 seedlings from an EMS induced population (27) (Fig. I). These plants represent three nonallelic dwarfing genes: *dwf1* (dwarf-1), *dwf2* (dwarf-2), and *dwf3* (dwarf-4). A fourth mutant, dwarf-8 (*dwf2^i*) is allelic to *dwf2*, yet has an intermediate phenotype.

Stem and leaf measurements were made to determine differences among dwarfs (Fig. 2). Normal (E-1) plants grew

Figure 1. Dwarf and normal plants 52 d after sowing. The early maturing strains E-1, *dwf2*, *dwf3*, and *dwf2^i* are flowering while the intermediate strains, E-3 and *dwf1*, are roughly 10 d away from flowering.

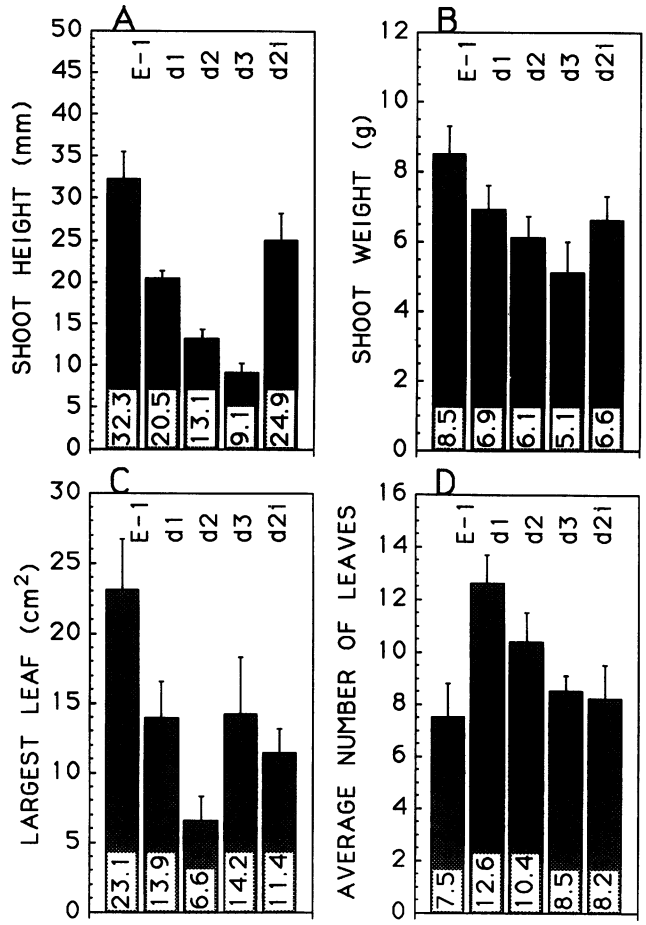

Figure 2. Total shoot height (A), shoot weight (B), single largest leaf (C), and average number of leaves per plant (D) measured at 63 d after sowing (single largest leaf was measured at 49 d). *dwf1* (E-3 type) was grown at a later date under similar conditions and was measured at 78 d. E-1 = E-1, *dwf1* = d1, *dwf2* = d2, *dwf3* = d3, and *dwf2*^{i} = d2i. Each value represents the average of five plants ± SD.

more than three times as tall as the shortest dwarf (*dwf3*) at maturity (63 days) (Fig. 2A). *dwf2*^{i} was the tallest dwarf growing to roughly twice the height of the allelic *dwf2*.

Shoots of E-1 weighed significantly more than those of the dwarfs at maturity (Fig. 2B). Despite the taller stature exhibited by *dwf1* and *dwf2*^{i} over the other dwarfs, these differences were not reflected in their weights, and were not significantly different from the other dwarfs. There were no significant differences between *dwf2*^{i} and *dwf2*. *dwf1* was the heaviest dwarf due in part to its later maturity and larger panicle size (data not shown).

Despite the very short stature and reduced weight of *dwf3*, this dwarf produced the largest leaves among the dwarfs (Fig. 2C). The area of the largest nonsenescing leaf was highest for *dwf3*, followed by *dwf1* and *dwf2*^{i}. Leaf areas for these three dwarfs were significantly larger than *dwf2*.

dwf1 and *dwf2* had a greater total number of leaves per plant than the other plants (Fig. 2D). For *dwf1* this could be related to its later maturity. *dwf2* has the same maturity E-1 and therefore may have an altered plastochron.

Light Microscopy

Cross-sections of *dwf2* leaves were nearly twice the thickness and had nearly twice the number of palisade and mesophyll layers compared to E-1 leaves (Table I). Leaf epidermal cells of *dwf2* were about one-half the size of E-1 cells and dwarf stomata were less than half the size of the E-1 stomata. However, there were equal numbers of cells per total leaf area for E-1 and dwarf.

Epidermal cells were also measured for E-1 and *dwf2* hypocotyls and similar results were obtained (Table I). Despite their greatly reduced size, total numbers of exterior cells on dwarf hypocotyls were nearly equal when calculated on an area basis.

Mature morphological comparisons of E-1 and *dwf2* showed that dwarf flowers had misshapen petals, reduced and swollen stigmas, and reduced and swollen pollen tube structures compared to E-1 (data not shown).

Table I. *Anatomical Comparisons between dwf2 and E-1 Lettuce*

Cell counts were taken from hypocotyl and leaf epidermal surfaces as well as leaf cross-sections for E1 and dwarf-2. They were examined with a light microscope. Values represent averages of 5 samples ± SD.

Comparisons	E-1	*dwf2*
Leaf Cross-Sections		
Total thickness	0.09 mm ± 0.02	0.16 mm ± 0.03
Palisade parenchyma	1 to 2 layers	3 layers
Mesophyll cells	5 to 6 layers	9 layers
Cell counts		
Leaf epidermal cell area	0.185 mm² ± 0.02	0.119 mm² ± 0.016
Cells per total leaf area	52,594 cells ± 1,832	52,773 cells ± 2,524
Leaf stomatal area	1.25 mm² ± 0.47	0.53 mm² ± 0.14
Stomata per leaf area	7,784 stomata ± 544	9,849 stomata ± 728
Hypocotyl epidermal cells	0.021 mm² ± 0.0047	0.013 mm² ± 0.0021
Cells per hypocotyl area	46,333 cells ± 3,132	48,307 ± 2,846

Seedling Growth

The growth of 6-d-old seedlings, grown in Petri dishes under controlled conditions, was measured to characterize growth by the different dwarfs (Fig. 3). As light intensity was decreased, hypocotyl lengths increased.

E-1 exhibited the most hypocotyl growth under both the high and low light regimes (Fig. 3A). *dwf2* seedlings had the shortest hypocotyls under high and low light conditions.

In contrast, roots were significantly longer for dwarfs than for E-1 (Fig. 3B) and exhibited an inverse relationship with hypocotyl length. Only *dwf3* roots under low light were shorter than E-1. Hypocotyl length was plotted *versus* root length for dwarf and E-1 seedlings to determine the extent of an apparent inverse relationship between the growth of these two organs. Under high light conditions, there was clearly a strong negative correlation ($r = -0.71$): as root length increased, hypocotyl length decreased. However, under low light conditions, there was no relationship ($r = -0.13$).

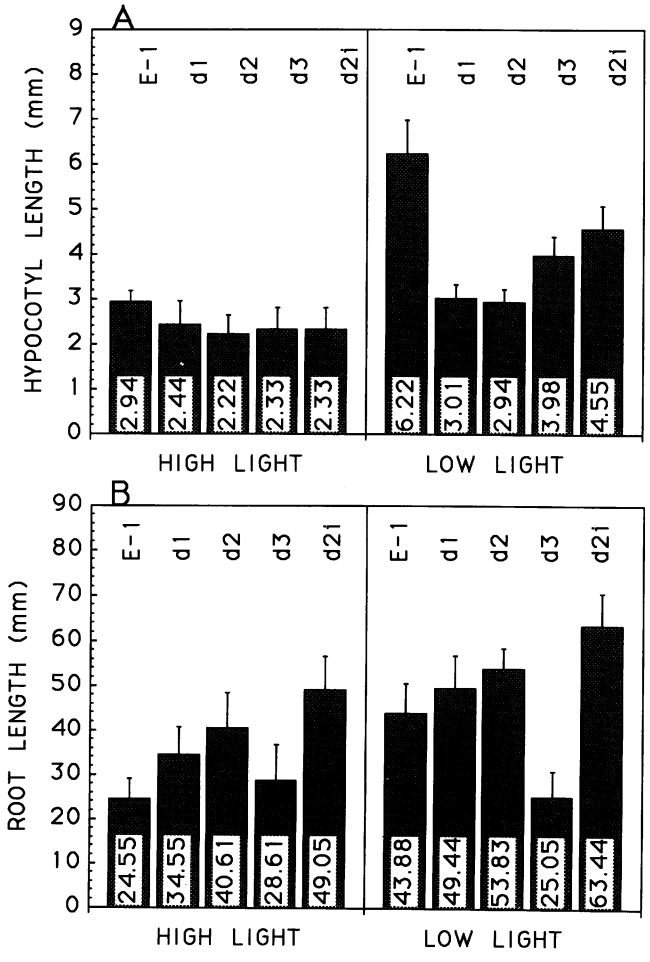

Figure 3. Intact hypocotyls (A) and roots (B) measured on 6-d-old seedlings. Seedlings were grown under continuous light at two different light intensities (120 μmol m^{-2} s^{-1} and 45 μmol m^{-2} s^{-1}). E-1 = E-1, *dwf1* = d1, *dwf2* = d2, *dwf3* = d3, and *dwf2^i* = d2i. Each measurement is an average of 20 seedlings ± SD.

Response to GA_3

With spray applications of 10 μM GA_3 twice weekly, all the dwarfs could be made to resemble normal E-1 phenotypes (data not shown). In the seedling stage, normal and dwarfs responded to GA_3 treatment with increased hypocotyl length (Fig. 4A). Hypocotyls of each seedling group exhibited elongation proportional to the log of the GA_3 concentration up to 10 μM, except *dwf3*. Length of normal hypocotyls for all but *dwf3* seedlings were similar and did not differ statistically. All seedlings except *dwf3* germinated normally. *dwf3* has abnormally shaped seeds which appear shrunken and slightly twisted. These seeds germinate more slowly than either E-1 or the other dwarfs. All other hypocotyls were measured 108 h after imbibition, while *dwf3* was measured at 132 h. Thus, despite the later germination of *dwf3*, all strains of dwarf and normal lettuce were generally as responsive to applied GA_3 after imbibition.

At the rosette stage (21 d), all the dwarfs responded to GA_3, confirming the responsive nature of these mutants to exogenous GAs during a later stage of development. Stem elongation was proportional to the log of the GA_3 concentration (Fig. 4B). However, stems of the normal E-1 and *dwf2^i* responded to lower concentrations (≥0.01 μM) than the other dwarfs (≥1.0 μM). The capacity of the other dwarfs to respond to GA_3 was significantly less. The highest concentration (100.0 μM) inhibited elongation in E-1 while *dwf2^i* continued to elongate. The *dwf2^i* response and its intermediate stature suggest that in lettuce, height is in some way related to GA_3 responsiveness. This result is supported by results shown in Figure 4C. Leaf elongation confirmed that all dwarfs were less responsive to GA_3 than E-1, though among the dwarfs, *dwf2^i* was again most responsive.

Response to Members of the GA Biosynthetic Pathway

The intact lettuce hypocotyl bioassay was used to compare the responses of *dwf2* and E-1 to exogenous gibberellins. Because of the scarcity of the available intermediates, a mini-drop method was developed to minimize the solution volume. Preliminary attempts to adapt the rice seedling bioassay (13), which involves placing a small drop of GA solution on the growing seedling, produced erratic results in lettuce. A consistent response was obtained by allowing seeds to fully imbibe 10 μL droplets of GA solution placed on glass. During germination the radicle and cotyledons emerge, but the hypocotyl has not yet elongated. Upon transfer to moist filter paper in continuous light, the hypocotyls elongate in response to the GA it has imbibed.

ent-Kaurenol, *ent*-kaurenoic acid, and GA_{53}-aldehyde were inactive in promoting *dwf2* hypocotyl extension at all concentrations (Fig. 5A). In contrast, GA_1 and GA_{20} were active at low concentrations while GA_{53} and GA_{19} were active only at high concentrations. These results were very similar to those for E-1 plants (Fig. 5B) and confirm the general responsiveness of both dwarf and E-1 hypocotyls to GA_3 among all dwarfs (Fig. 4A).

These data show that the E-1 hypocotyl tissue is quite GA-responsive. They also suggest that GA biosynthesis is limiting in the E-1 hypocotyl tissue and that the metabolic lesion in

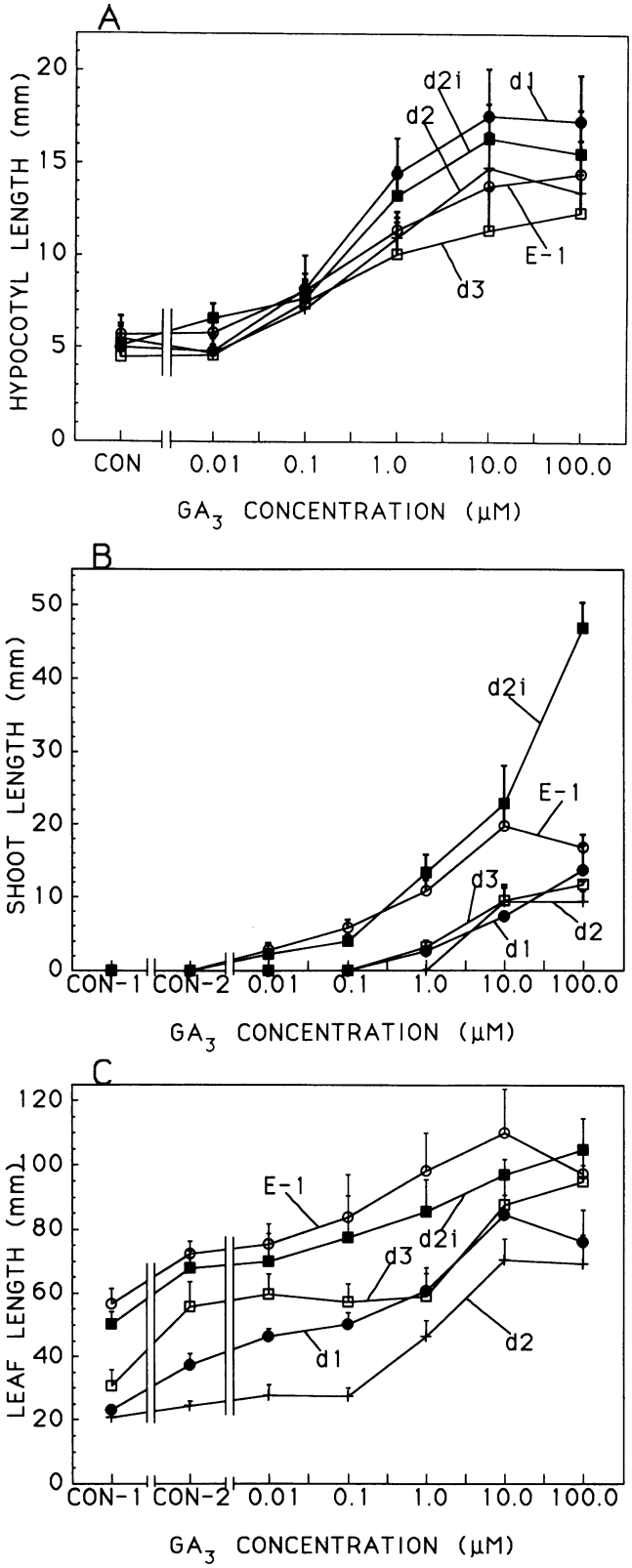

Figure 4. Response to increasing concentrations of GA_3 by intact hypocotyls (A), intact shoots (B), and intact leaves (C) of E-1, *dwf1*, *dwf2*, *dwf3*, and *dwf2*^{i}. E-1 (○), *dwf1* (d1 = ●), *dwf2* (d2 = +), *dwf3* (d3 = □), *dwf2*^{i} (d2i = ■). In A, *Con* represents seedlings grown without GA_3. In B and C, *Con-1* represents shoot heights at the time of GA_3 application. *Con-2* represents shoot heights of plants treated as a water control, measured the same time as the treated plants (1 week later). Each point represents the average of 20 seedlings (A) and five plants (B and C) ± SD.

dwf2 occurs at an early stage of GA-biosynthesis. However, these suggestions remain to be proven.

DISCUSSION

The dwarf mutants exhibited distinct anatomical and morphological features which we used to distinguish one line from another. Dwarfs were all smaller than E-1 except for their roots. The seedling root lengths of *dwf2* and *dwf2*^{i} were significantly longer than those of E-1. Since shoot/root ratios are dependent on resource allocation, the inverse relationship between hypocotyl growth and root growth suggests a possible role for GA (direct or indirect) in regulating nutrient translocation in lettuce. Accurate analysis at the mature stage is needed to confirm this observation.

Interestingly, *dwf2*^{i} was not simply a larger phenotype of the allelic *dwf2*. Whereas *dwf2*^{i} hypocotyl and mature shoot height as well as total leaf number were nearly that of E-1, total shoot weight and total leaf area averaged at or below the *dwf2* levels. *dwf2*^{i} root lengths were the longest. *dwf1* and *dwf3* had nearly the same size leaves as E-1, but a dwarfed stem. These two dwarfs appear to exhibit stem-specific dwarfing and would be of interest for future studies involving tissue specific responses to GAs.

Comparison of *dwf2* and E-1 at the light microscope level showed E-1 had fewer cells per unit surface area. However, the total numbers of cells were similar for dwarf and E-1 at both the hypocotyl and mature plant stages. These data suggest that the differences between dwarf and E-1 stature can be accounted for by differences in cell expansion, consistent with the findings of Stuart *et al.* (24).

Sachs and Lang (20) investigated the effect of GA_3 on elongating axes of the rosette plants *Hyoscyamus niger* L. and *Samolus parviflorus* Raf. They found that cell division was the major component of growth during elongation. In our study this question was not addressed, but a similar mechanism may also operate in lettuce stems.

Leaf cross-sections of *dwf2* were nearly twice the thickness of E-1 leaves. This supports a similar observation reported by Koornneef *et al.* (8) for GA-deficient mutants of tomato. There were also nearly 50% more leaves on *dwf1* and *dwf2*, suggesting at least for *dwf2* a higher rate of leaf initiation compared to E-1.

Mutants of maize, pea, and rice were described by Phinney (16) as being nearly exact dwarf variations of the wild-type form, except in the case of maize floral development. Photographs illustrating dwarfs of the same species have been published. Size variation between dwarf strains did not apparently vary for maize and rice (16), while differences were evident among mutants of pea (5), *Arabidopsis* (9), and tomato (8). This type of variation was also observed in our lettuce mutants (Fig. 1). Koornneef and van der Veen (10) reported their *Arabidopsis* dwarfs had poor petal and stamen

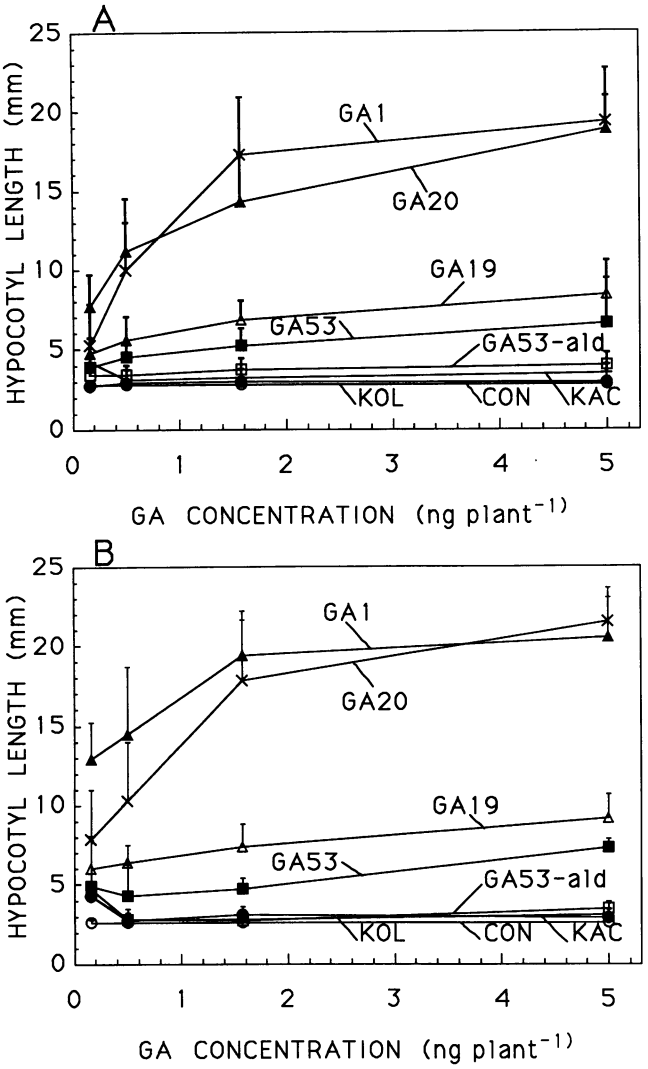

Figure 5. Intact hypocotyl elongation assay of *dwf2* (A) and E-1 (B) grown in the light, using GA biosynthetic pathway members at increasing concentrations. Control (CON = ○), *ent*-kaurenol (KOL = ●), *ent*-kaurenoic acid (KAC = +), GA_{53}-aldehyde (GA53-ald = □), GA_{53} (■), GA_{19} (△), GA_{20} (×), and GA_1 (▲). Each point represents an average (± SD) of two experiments, 20 seedlings per experiment.

development. This agrees with our observations of dwarf flowers.

In the hypocotyl stage, response of dwarf and normal lettuce to GA_3, as well as certain 13-hydroxy GAs, was quite similar. There was very little difference in responsiveness to applied GAs at this stage except that *dwf3* had delayed germination which is genetically controlled. However, at 21 d the dwarfs were less responsive to GA_3 than the normal genotype, and dwarf plants required higher concentrations of GA_3 to produce stem and leaf equivalents of normal plants. Here, the *dwf2*^{i} response indicates that plant height in lettuce may be associated with GA_3 responsiveness. Koornneef *et al.* (8) found the same for tomato. This suggests that there is more than simply decreased GA biosynthesis at work in these mutants.

The strong response by hypocotyls of normal lettuce to applied GAs has been commonly used for gibberellin bioassays. At least two other nondwarf bioassays are reported: cucumber hypocotyl and excised pea epicotyl (1, 23). Thus, a high degree of responsiveness in normal, wild-type lines is not unique to lettuce. Nevertheless, it is the reduced responsiveness of dwarf lettuce to applied GA during the rosette stage (21 d) which makes classification of these plants difficult as biosynthetic mutants. We have shown they are typical responsive mutants, and they can be restored to the E-1 phenotype with repeated sprays of 10 μM GA_3. They also can synthesize their own GAs as shown in Figure 3; dwarf seedlings became etiolated in response to decreased light levels. Responsiveness of dwarfs in other species to applied GAs tends to be higher than for the normal. While dwarfs of maize (15), *Arabidopsis* (9), rice (11), and barley (4) all elongated significantly more than their normal genotypes, dwarf pea (19) and tomato (8) responded less than their normals. The lettuce results concur with this latter group.

The correlation between biological activity and applied endogenous GAs and their precursors has been used to investigate the position of the metabolic blocks in mutants of maize (17), and of pea (6). Such bioassays were conducted with *dwf2* and E-1 lettuce seedlings. In both cases, a break in biological activity between the *ent*-kaurenoids and GA_{53} was observed. *Per se,* these results with *dwf2* do not establish that the *dwf2* mutation occurs prior to GA_{53}. However, the fact that *dwf2* and E-1 seedlings do respond similarly to the applied GAs in the hypocotyl elongation assay may indicate that the biochemical step affected in *dwf2* occurs early in the GA biosynthetic pathway.

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